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| <p>(21) International Application Number: PCT/US00/06718 (22) International Filing Date: 12 May 2000 (12.05.00) (30) Priority Data: 60/134,055 13 May 1999 (13.05.99) US (71) Applicants (for all designated States except US): EXPONENTIAL BIOTHERAPIES, INC. [US/US]; 150 Main Street, Port Washington, NY 11050 (US). NATIONAL INSTITUTES OF HEALTH [US/US]; Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MERRIL, Carl, R. [US/US]; National Institute of Mental Health, Building 10, Room 2D54, 9000 Rockville Pike, Bethesda, MD 20892 (US). CARLTON, Richard, M. [US/US]; 150 Main Street, Port Washington, NY 11050 (US). ADHYA, Sankar [US/US]; 14400 Kings Grant Street, Gaithersburg, MD 20878 (US). (74) Agents: MURRAY, Robert, B. et al.; Arent Fox Kintner Plolkin & Kahn, PLLC, Suite 600, 1050 Connecticut Avenue, N.W., Washington, DC 20036-5339 (US).</p> | | <p>(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p> |
| <p>(54) Title: STRAINS OF BACTERIOPHAGE USEFUL FOR RESCUING PATIENTS INFECTED WITH VANCOMYCIN-RESISTANT ENTEROCOCCUS FAECIUM</p> <p>(57) Abstract</p> <p>The present invention involves the use of specific phages, designated ENB6 and ENB13, that kill many clinical isolates of vancomycin-resistant <i>Enterococcus faecium</i> and of vancomycin-sensitive <i>Enterococcus faecium</i>. The genome of one of the phage strains, ENB6 has been partially sequenced, and is shown to not contain nucleotide sequences for known bacterial virulence genes or for the vancomycin resistance cassette. Its efficacy in rescuing mice from otherwise-fatal bacteremias is documented herein.</p> | | |

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**Strains of Bacteriophage Useful for Rescuing Patients Infected With
Vancomycin-Resistant *Enterococcus Faecium***

FIELD OF THE INVENTION

Several clinically important species of bacteria have become multidrug resistant ("MDR"). One of these is Enterococcus faecium, a commensal that does not cause disease in its habitual niche (the intestines) but which can breach the gut barrier and cause bacteremias if the immune system fails to eliminate the bacteria.

5 Immunocompromised patients cannot eliminate these bacteria, and deaths in such patients are becoming increasingly commonplace.

As E. faecium acquired resistance to increasing numbers of antibiotics (e.g. penicillins, cephalosporins and aminoglycosides), treatment options became progressively narrowed until vancomycin was the drug of last resort. In 1989 the

10 first clinical isolates of vancomycin-resistant E. faecium (VREF) were reported. Physicians were then confronted with a pathogen that was difficult and often impossible to treat. In recent years the prevalence of these vancomycin-resistant strains has increased to the point that hospitals typically report that approximately 40% of the E. faecium clinical isolates are vancomycin resistant. Correspondingly,

15 fatal bacteremias are being reported in steadily increasing numbers.

While the pharmaceutical industry does introduce new antibiotics from time to time, it has become commonplace that new antibiotics become rapidly resisted by multidrug resistant ("MDR") bacteria. For example, Synercid® recently entered

the market as a treatment for vancomycin-resistant bacteria, including VREF. Resistance to this antibiotic began to appear even while it was in clinical trials, and by the time it was approved for commercial sales approximately 20% of VREF clinical isolates were reported fully resistant to this new antibiotic. The reason that MDR bacteria are so efficient at resisting newer antibiotics (even those to which they have never been exposed) is that the resistance mechanisms they've acquired enable them to defeat many different classes of antibiotics. For example, a mutant efflux pump can transport out many classes of drugs; and a mutation in the ribosomal subunit targeted by antibiotics can defeat several classes of drugs. An alternative to antibiotics is therefore needed to control such MDR bacteria.

Bacteriophage (phage) therapy offers one such alternative. The present invention describes several examples of phage strains (for example ENB6) that rescues mice from a fulminant VREF bacteremia.

BACKGROUND OF THE INVENTION

As described in US Patent No# 5,688,501 by Merrill et al (and incorporated by reference herein), phage therapy of human bacterial infections failed for a number of technical reasons. One of the technical reasons was that phages tend to be rapidly cleared from the systemic circulation by the filtering action of the organs of the reticulo-endothelial system (RES). This rapid clearance prevents the phages from remaining in circulation long enough to reach and infect the target bacteria infecting the patient.

The above-cited invention solved the problem of rapid clearance by introducing a novel approach called "serial passage". In that technique, a large number of phages of a wild-type strain are injected into an animal, blood samples are taken at various intervals, and any phage particles still remaining in circulation at the time of the venipuncture will be present therein and can be grown to high titer on the host bacteria. This technique therefore selects for phage variants whose surface coat proteins are not readily detected by the RES, and such variants are amplified by cloning at the end of each round of serial passage. Since the phages being selected must be able to produce plaques on the lawn of the host bacteria, the technique also selects for those mutants that retain their ability to lyse the target bacteria. Finally, the long-circulating phage mutants obtained thereby were superior to the wild-types from which they were derived, in terms of rescuing an animal from an otherwise-fatal bacteremia. In the above-referenced patent, the bacterial target was a strain of E. coli, and the wild-type phage strain used was lambda coliphage.

In the present invention, phage strains that attack VREF hosts have been discovered by the present inventors. These strains were discovered through screening samples of sewage from the waste management system of Montgomery County, Maryland..

SUMMARY OF THE INVENTION

Phage strains were grown by standard techniques known in the art, by plating them on clinical isolates of VREF which were obtained from hospitalized

patients (with no identifiers as to the name of the patients). These stains are lytic when propagated in many clinical isolates of VREF.

These phage strains were grown to high titer, and they were characterized and defined through the methods described below using the phage strain ENB6 as an example.

DETAILED DESCRIPTION OF THE INVENTION

Details on the characterization of and host range of phage ENB6 are provided in this section. Details on the phage's utility, in terms of rescuing animals from an otherwise-lethal bacteremia, are provided in the section that follows.

1. Genomic sequencing

50 mg of phage ENB6 DNA was sheared and then random fragments were "shotgun cloned" into an M13-based vector for sequencing. The raw data was pre-screened and then the individual sequences were compiled into overlapping contigs.

The ENB6 genome contains at least 120 kb of DNA as determined by sequencing and gel electrophoretic analyses of extracted DNA. A total of 94.4 kb of nucleotide sequence has been defined at 99% confidence, while 24.7 kb has

been defined at a lower level of confidence. The remaining amount is presently undefined.

2. Analyzing the phage's genome for nucleotide sequences of interest, using
homology searches on databases as well as PCR probes

5 The ENB6 nucleotide sequences have been compared to all genes and
proteins registered in the databases using two alignment algorithms, BLASTN
(nucleotide sequence comparisons) and BLASTX (putative amino acid sequence
comparisons). All alignments of high confidence matched genes and gene products
of other bacteriophages including those for head, tail, polymerase and lysin
10 proteins. No extensive and significant match was found at the nucleotide or
predicted protein level to recognized whole genes of bacterial factors for
pathogenicity, infectivity, invasion, attachment or antibiotic resistance. However,
four short and dispersed alignments to these kinds of undesirable factors were
found as shown in Figure 1 and Table 1. The fraction of each protein exhibiting
15 some similarity to a potential gene product from ENB6 is not greater than 30 % in
any example, meaning, at best, only a partial gene exists. The short lengths of
identity suggest that only a subtle similarity exists at the amino acid sequence level.
If actually translated into protein products, these fragmented domains would either
be not functional or unfamiliar.

20 Thus, we find no evidence of whole genes for potentially hazardous factors
in the known nucleotide sequence of phage ENB6. Understanding that only part of

Table 1. Undesirable proteins found by BLASTX alignments of theoretical proteins derived from ENB6 nucleotide sequence.

| Source of query sequence | Target protein found to have some alignment | Alignment scores: identity per length (%), gaps per length | Fraction of target aligned |
|--------------------------|--|--|----------------------------|
| Contig 34 | Plasminogen binding protein (class C <i>Streptococci</i>) | 32/108 (29%), 13/108 | 61/454 (13 %) |
| Contig 37 | Orf1 protein of insertion element IS232 | 14/30 (46%) | 29/431 (7 %) |
| Contig 43 | Hemagglutinin (Influenza A virus) | 17/41 (41%) | 116/566 (20 %) |
| Contig 49 | orf14 protein of transposon Tn916 | 37/124 (29%), 6/124 | 96/329 (29 %) |

the genome was screened by database searches, we have undertaken a second approach to inspecting the ENB6 phage for potentially undesirable genes. We have designed oligonucleotide primers for physical screening of the phage DNA by PCR amplification. The genes searched are listed in Table 2.

- 5 Thus we have used sequence alignment searches and physical tests for known genes to address the concern for a potential risk of horizontal gene transfer through the therapeutic use of bacteriophage phage ENB6.

3. Electron microscopic study

Figure 2 is an electron microscopic picture of phage ENB6.

- 10 The routes of administration include but are not limited to: oral, aerosol or other device for delivery to the lungs, nasal spray, intravenous, intramuscular, intraperitoneal, intrathecal, vaginal, rectal, topical, lumbar puncture, intrathecal, and direct application to the brain and/or meninges. Excipients which can be used as a vehicle for the delivery of the phage will be apparent to those skilled in
- 15 the art. For example, the free phage could be in lyophilized form and be dissolved just prior to administration by IV injection. The dosage of administration is contemplated to be in the range of about 10^3 to about 10^{13} pfu/per kg/per day, and preferably about 10^{12} pfu/per kg/per day. The phage are

Table 2. Proteins screened by PCR amplification of ENB6 DNA.

| Genes Targeted for amplification by PCR | Source and Description |
|---|--|
| <i>cytL1, cytM, cytB, cytA</i> | Cytolytic genes contained on the conjugative (transferable) plasmid pAD1 of <i>E. faecalis</i> . |
| 5 <i>traC</i> | Hemolytic bacteriocin from pAD1 of <i>E. faecalis</i> . |
| <i>pneu</i> | Pneumolysin from <i>S. pneumoniae</i> . |
| <i>sly</i> | Cytolytic toxin from <i>Streptococcus suis</i> . |
| <i>slo</i> | Streptolysin O from plasmid pMK157 <i>Streptococcus canis</i> . |
| 10 <i>slo</i> | Streptolysin O from group A, C and G <i>Streptococci</i> . |
| <i>sagC</i> | Streptolysin S |
| <i>L50</i> | Enterocin L50 from <i>E. faecium</i> . |
| <i>aph</i> | Resistance to aminoglycoside antibiotics (gentamycin, kanamycin) from <i>E. casseliflavus</i> . |
| <i>genta</i> | Newly characterized resistance gene to gentamycin from <i>S. aureus</i> . |
| <i>ermAM</i> | Resistance to erythromycin from plasmid pAM-b-1 of <i>S. faecalis</i> . |
| 15 <i>ery</i> | Resistance to erythromycin from transposon Tn917. |
| <i>penA</i> | Class AmpC β -lactamase from <i>S. pneumoniae</i> giving resistance to penicillin antibiotics. |
| <i>orf14</i> | Orf14 protein of transposon Tn916 of <i>E. faecalis</i> . |
| <i>orf1</i> | Orf1 protein of insertion sequence (mobile DNA element) IS232 of <i>B. thuringiensis</i> . |
| <i>tetM</i> | Tetracycline Resistance from transposon Tn916 of <i>E. faecalis</i> . |
| 20 <i>esp</i> | Surface protein of virulent <i>Enterococci</i> clinical isolates. |

administered until successful elimination of the pathogenic Enterococcus
faecium is achieved.

As used in the present application, the term "substantially reduce"
indicates that the number of bacteria is reduced to a number which can be
5 completely eliminated by the animal's defense system or by using conventional
antibacterial therapies.

The present invention will be particularly useful in treating critically ill
patients or those with severe underlying disease or immunosuppression (e.g.
patients in ICUs or in oncology or transplant wards), patients who have had an
10 intraabdominal or cardio-thoracic surgical procedure or an indwelling urinary or
central venous catheter, and persons who have had a prolonged hospital stay or
received multi-antimicrobial and/or vancomycin therapy.

Deposits of ENB6 (ATCC # PTA-40) and ENB13 (ATCC # PTA-39) were
made on May 12, 1999 at the American Type Culture Collection, 10801
15 University Blvd., Manassas, VA. 20110-2209.

The foregoing embodiments of the present invention are further described
in the following Examples. However, the present invention is not limited by the
Examples, and variations will be apparent to those skilled in the art.

EXAMPLES

1. VREF Bacteremia Rescue Experiment #1: Dose-Finding Study

Figures 3 and 4 show the results of a dose-finding study.

Materials and Methods:

5 We had previously determined that the $2xLD_{50}$ dose for a clinical VREF isolate designated CRMEN44 is 1×10^9 CFU, when injected I.P. into one month-old balb/c female mice. In other studies (data not shown here), we had determined that the I.P. injection of this bacteria strain causes a bacteremia within 15 minutes, and that the I.P. injection of phage ENB6 causes a viremia within 15 minutes. In this study,
10 the following dosages of phage ENB6 were administered once (and only once) I.P., exactly $\frac{1}{2}$ hour after the bacterial challenge: 3×10^9 , 3×10^8 , 3×10^6 , and 3×10^4 PFU plaque forming units (PFU). In addition, a dose of 3×10^9 PFU was administered I.P. to another set of animals, as a control, with no bacterial challenge.

The non-parametric rating scale for observable signs of illness is as follows:

15 5 = Normal animal; 4 = Mild lethargy; 3 = Mild lethargy + Ruffled fur; 2 = the above, plus exudate around the eyes; 1 = Moribund; and 0 = Dead.

Results:

Phage administered as a control did not produce any detectable symptoms in the animals. Bacteria administered without any phage treatment caused the death of
20 all the animals, within 48 hours. With the two highest dosages of phage there were

no deaths, and the animals recovered within 24 hours from the minimal signs of illness that had developed, with no relapse over a period of 21 days of observation. While there were some deaths with the two lowest dosages of phage, nevertheless roughly half the animals in these groups survived (and recovered completely) after becoming moderately ill.

Discussion:

Phage ENB6 rescues animals from an otherwise-fatal dose of VREF, a bacterial pathogen for which no consistently reliable antibiotic is currently available. The infection here is fulminant, using a concentration of bacteria (10^9 , which will be very concentrated in the 3 ml of blood in a mouse's circulatory system) that is orders-of-magnitude greater than that found in bacteremic humans (where titers in blood reach only 10^2 to 10^4 CFU per cc).

Conclusion:

While an IND approval will be required from the FDA before such phages can be administered therapeutically to humans, phage strains that lyse VREF hosts *in-vitro* should be able to kill the bacterial targets wherever encountered (*in-vivo*), whether within the mouse or the human circulatory system. Moreover, multiple phage doses will be employed in treating humans. In this experiment only one dose was administered, in order to demonstrate the ability of the phages to grow exponentially in number and to thereby overwhelm the target bacteria.

2. VREF Bacteremia Rescue Experiment #2: Delayed Treatment

Figures 5 and 6 show the results of delay in the treatment of a fulminant bacteremia.

Materials and Methods:

- 5 Same as in Experiment 1, except for the dosage and timing of the phage administration. In this experiment, only the highest dose (3×10^9) of phage ENB6 was administered. After the I.P. bacterial challenge, the one (and only one) I.P. administration of the phage dose was delayed until one or another of the following time points: 2, 5, 8, 14, 18 and 24 hours. One group of animals received no phage
10 treatment, as a control.

Results:

- With no treatment, all animals were dead within 48 hours. With treatment delayed 2 hours and 5 hours, all animals survived (after becoming moderately ill). With treatment delayed from 8 – 24 hours approximately half the animals died, but for the
15 half that survived, even though the degree of illness reached was severe, nevertheless there was full and complete recovery by day 4 or 5, with no relapse.

Discussion:

Even when treatment of a fulminant bacteremia in mice is delayed, phage ENB6 tends to rescue the animals from an otherwise-fatal dose of VREF. The rescue is

100% with delays up to and including 5 hours. With delays between 8 – 24 hours, approximately 50% of the animals survive and go on to recover completely.

Conclusion:

While an IND approval will be required from the FDA before such phages can be administered therapeutically to humans, phage strains that lyse VREF hosts *in-vitro* should be able to kill the bacterial targets wherever encountered (*in-vivo*), whether within the mouse or the human circulatory system. In the human, concentrations of VREF are orders-of-magnitude lower than the concentrations achieved here, so it should be that much easier to achieve a therapeutic success. Moreover, in treating humans, multiple administration of phage will be employed. In this experiment only one dose was administered, in order to demonstrate the ability of the phages to grow exponentially in number and to thereby overwhelm the target bacteria.

We claim:

1. A wild-type phage which is lytic for strains of vancomycin-resistant *Enterococcus faecium* (VREF) as well as for strains of vancomycin-sensitive *Enterococcus faecium* (VSEF), wherein said phage is selected from the group
5 consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39)

2. A method for treating an *Enterococcus faecium* infection comprising administering an amount of a phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) effective to eradicate or substantially reduce an *Enterococcus faecium* infection to a patient in need of such
10 treatment.

3. The method according to claim 2, wherein said *Enterococcus faecium* is vancomycin-resistant *Enterococcus faecium*.

4. The method according to claim 2, wherein said phage is administered by a route selected from the group consisting of orally, topically, intravenously, intra-
15 arterially, intraperitoneally, intrathecally, by inhalation, by nasal spray, by irrigation of a wound, by suppository, and by enema.

5. The method according to claim 2, wherein said phage is administered at a total dose of between $10^3 - 10^{12}$ PFU.

6. The method according to claim 5, wherein said phage is administered at a total dose of between $10^5 - 10^{11}$ PFU.

5 7. The method according to claim 2, further comprising administering an antibiotic.

8. A method for reducing the probability of an *Enterococcus faecium* colonization becoming an infection comprising administering an amount of phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC#-
10 PTA-39) effective to reduce the probability of such colonization becoming an infection to a patient at risk for an *Enterococcus faecium* infection.

9. The method according to claim 8, wherein said phage is administered by a route selected from the group consisting of orally, topically, intravenously, intra-arterially, intraperitoneally, intrathecally, by inhalation, by nasal spray, by irrigation
15 of a wound, by suppository, and by enema.

10. The method according to claim 8, wherein said phage is administered at a total dose of between $10^3 - 10^{12}$ PFU.

11. The method according to claim 10, wherein said phage is administered at a total dose of between 10^5 – 10^{11} PFU.
12. A pharmaceutical composition comprising a phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) in combination with a pharmaceutical carrier.
13. The composition according to claim 12, further comprising an antibiotic.

Figure 1. Alignments of hypothetical amino acid sequences predicted from ENB6 DNA with the protein databases.

BLASTX alignments of the highest score (Expect Value less than 0.005) to proteins of potentially undesirable factors are shown. "Query" = putative protein from ENB6; "Sbjct" = target protein found in database. Numbers = codon or amino acid number; {} = total length of target protein.

1. Plasminogen-binding protein MLC36 from group C *Streptococcus* sp.

```

Query: 70  RTWTEYLATGHVHDKNHAKQLERLSKRDISLGDVATVVD FMSRRNDGYITALIEQNSVNE
249
      R  T      T +V +K  A +LE+L   +++  D  ++VD M   ND   T   +   + +
Sbjct: 36  RLVTNMWKTQYVKEKQRADELEKLLHSEVA-DYNSLVDMKMKVVNDSLQTTKRDYEEIEK
93

Query: 250  KLFNKL----GVTDKMRNEAKA-----EYEVELKQAQEEIKKLQEELAEKLQKGE*Y
393
      +L NKL      + +K++N+   +           E +++L Q   +   L+ EL ++ QK E
Sbjct: 94  ELGNKLKENQDLEEKLNKEFSLGEALRYINELDLKGLQLNIDNIDLKHELEQEKQKAEAY
154 {454}

```

2. IstA protein homolog from *Bacillus thuringiensis* similar to ORF1 protein of insertion sequence IS232

```

Query: 871  QFAYDFAFSGYPQLAGMPPSSGQVDAPQMI 960
      QFA DF F   P +AG P + G+V+AP   +
Sbjct: 239  QFAQDFGFKVQPCIAGRPNTKGKVEAPMKL 268 {431}

```

3. Hemagglutinin protein from Influenza A virus

```

Query: 2636 NQAVLARNREFNKKIQREGAYLDHLIEGLKEHLSEE-----LENTNTLKYE
2499
      N+ +   N +F++I++E + ++   I+ L++++ +           LEN NT+   +
Sbjct: 398  NRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQNTIDLTD
457

Query: 2498 PELRVKGKPSDREMILCLSDWHIGAF-----VNNIDTGGYNYDIFR-ERLNS
2361
      E+   + + R++   D   G F           + +I G YN+DI+R E LN+
Sbjct: 458  SEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIRNGTYNHDIYRDEALNN
514 {566}

```

4. *Bacillus subtilis* protein similar to ORF14 of *E. faecalis* transposon TN916

```

Query: 2523 YDWGGGRTGRDPFESSPIATDCSSFWWC FKHAGVELNGGATGMTTWSIIADTKLETIAT
2344
      Y WGG      +           DCS V W F AG+ L   A
Sbjct: 224  YAWGGS-----NPETGFDCSGLVQWSFAKAGITLPRTAQEQ-----
259

```

Query: 2343 RGQKNSAIFDKMKAGDIIWF-----RNCEHIGIYCGEGKMVACNGSGNMNESPTAGIIV
2182

G + AGD+++F + H+GIY G G+M N SG I
Sbjct: 260 HGATKKISEKEATAGDLVFFGGTYEGKAITHVGIYVGNGRMFNSND SG-----IQY
310

Query: 2181 SDMTSGYWWD 2152
SD+ SGYW D

Sbjct: 311 SDLKSGYWRD 320 {329}

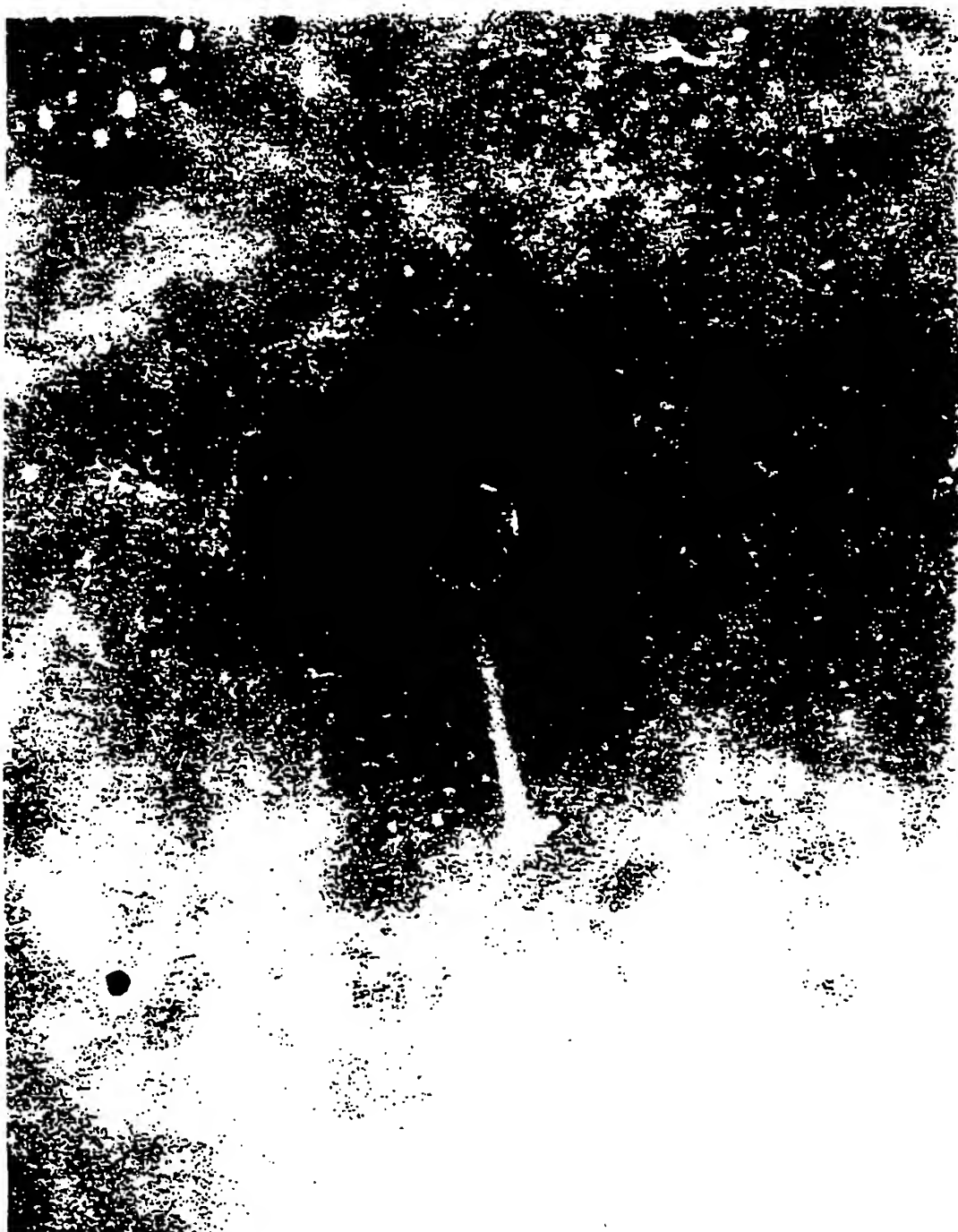


Figure 2

Effect of Phage Concentration on VRE Infected Mice

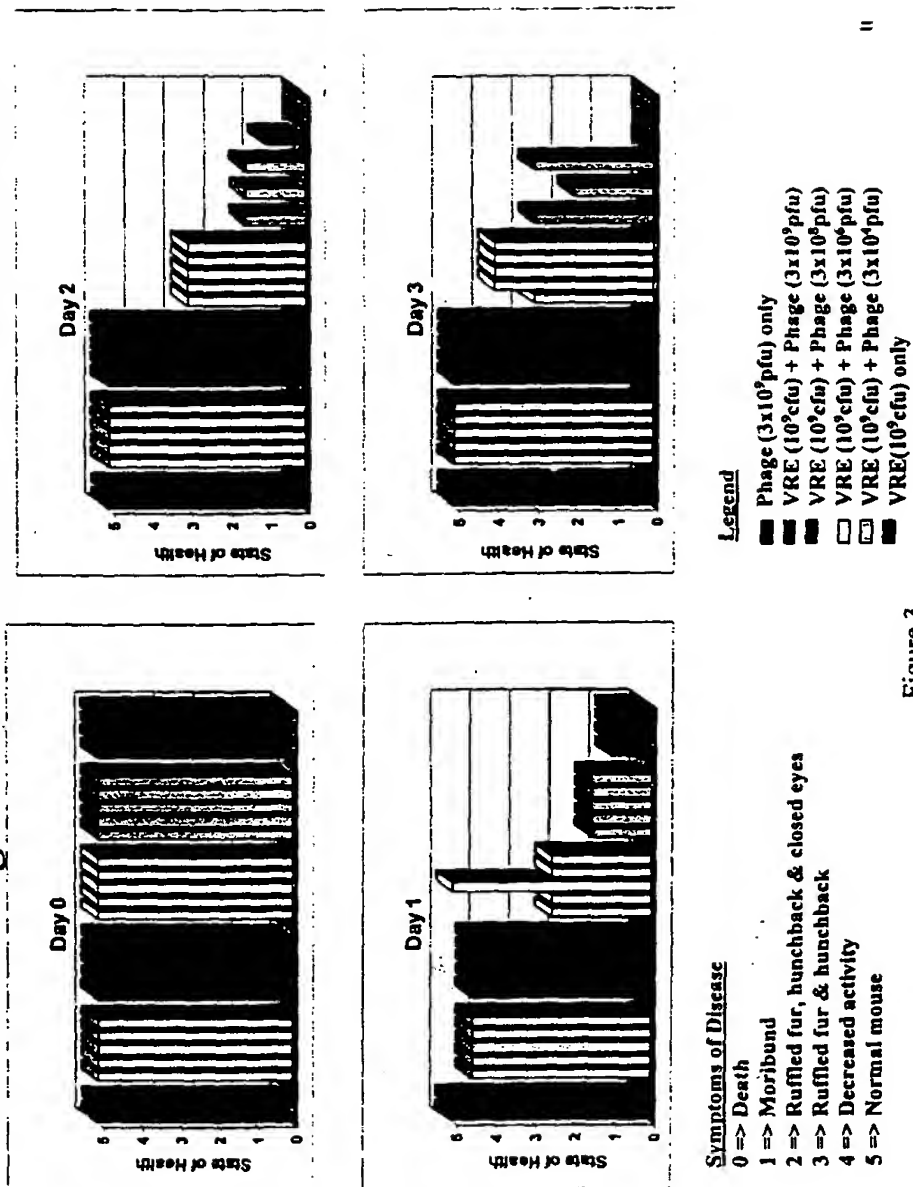


Figure 3

Effect of Phage Concentration on VRE Infected Mice



Figure 4

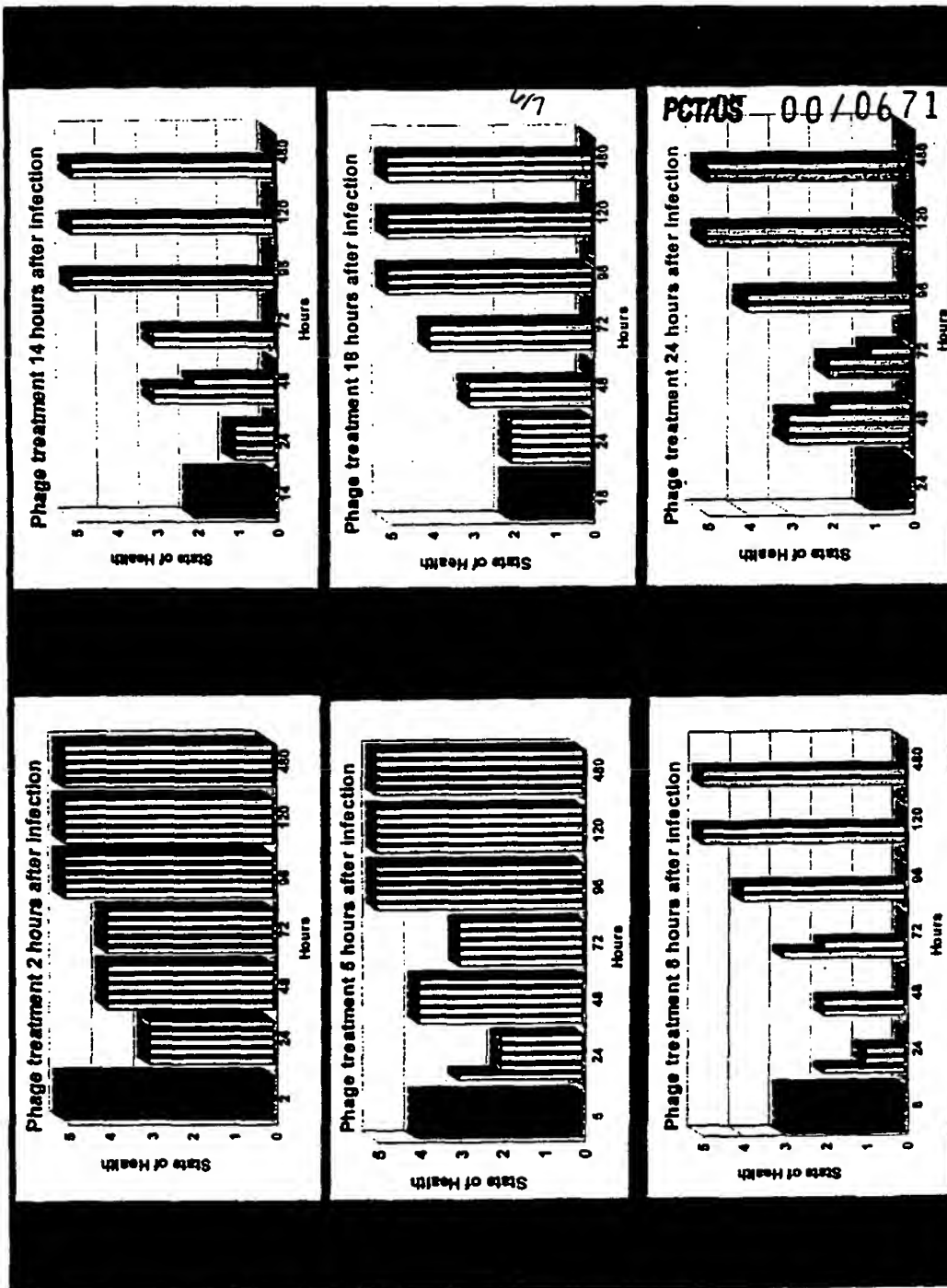


Figure 5

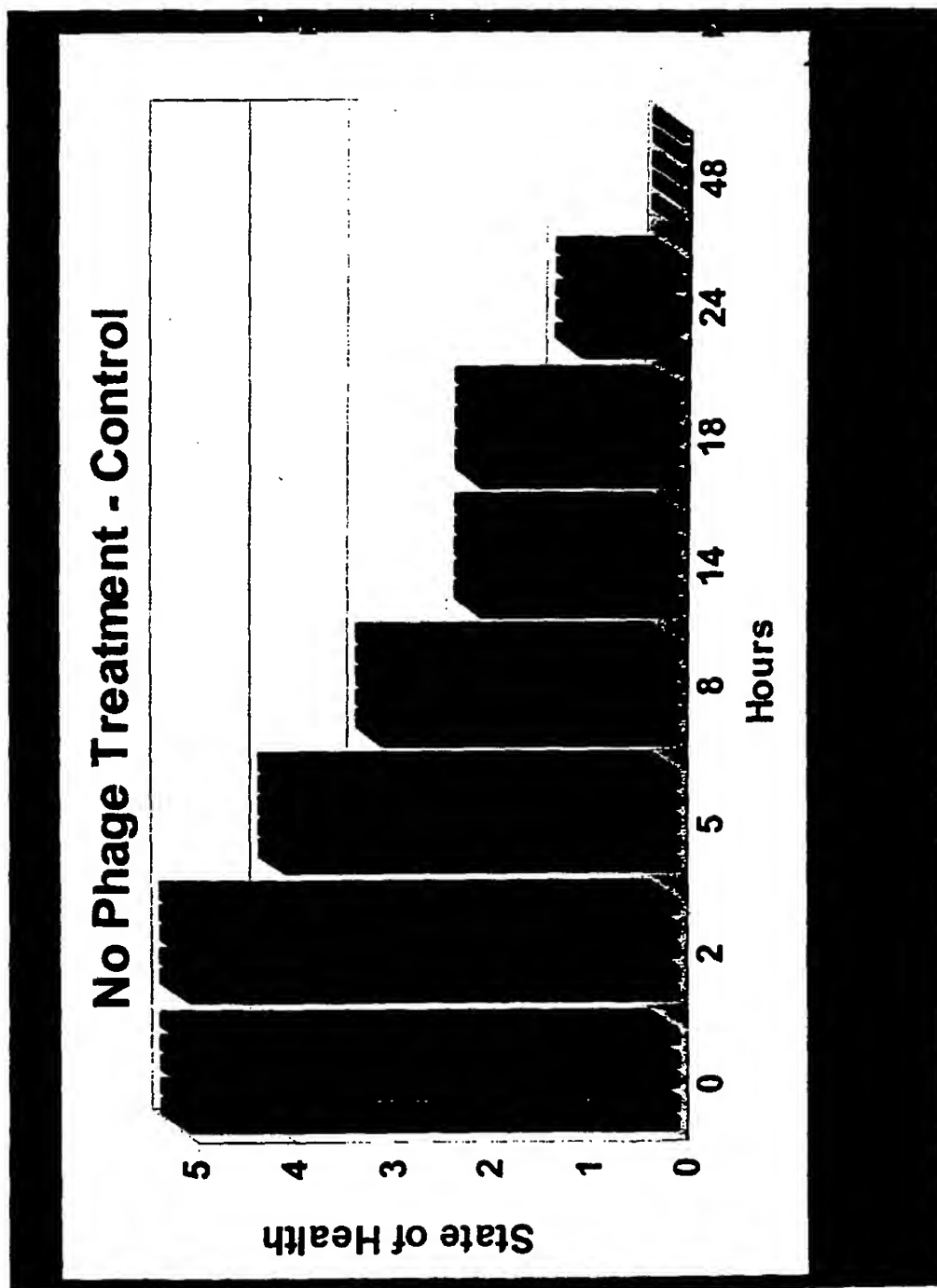


Figure 6